Coupling of ferric iron spin and allosteric equilibrium in hemoglobin

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ABSTRACT The allosteric transition in triply ferric hemoglobin has been studied with different ferric ligands. This valency hybrid permits observation of oxygen or CO binding properties to the single ferrous subunit, whereas the liganded state of the other three ferric subunits can be varied. The ferric hemoglobin (Hb) tetramer in the absence of effectors is generally in the high oxygen affinity (*R*) state; addition of inositol hexaphosphate induces a transition towards the deoxy (*T*) conformation. The fraction of *T* -state formed depends on the ferric ligand and is correlated with the spin state of the ferric iron complexes. High-spin ferric ligands such as water or fluoride show the most *T*-state, whereas low-spin ligands such as cyanide show the least.

The oxygen equilbrium data and kinetics of CO recombination indicate that the allosteric equilibrium can be treated in a fashion analogous to the two-state model. The binding of a low-spin ferric ligand induces a change in the allosteric equilibrium towards the R-state by about a factor of 150 (at pH 6.5), similar to that of the ferrous ligands oxygen or CO; however, each high-spin ferric ligand induces a *T* to *R* shift by a factor of 40.

INTRODUCTION

The influence of the spin state of the heme-ligand complex on the allosteric equilibrium in ferric hemoglobin (Hb) was suggested by Perutz, based on changes in the absorption spectra (Perutz et al., 1974) and oxidation potentials (Kilmartin, 1973), and supported by the fact that metHb (aquo- or cyano-) crystallizes in the liganded (*R*-state) conformation (Deatherage et al., 1976), whereas fluoro-metHb with inositol hexaphosphate (IHP) shows a ferrous deoxy (*T*-state) structure (Fermi and Perutz, 1977). Additional work has characterized the quaternary change in metHb (Olson, 1976; Noble et al., 1989); however, studies on completely ferric Hb do not permit a comparison with the usual oxygen binding properties, as oxygen does not bind to the ferric species. We have prepared ferric hemoglobin at high oxidation levels to study oxygen or CO binding to the single remaining ferrous subunit. In this way, different ferric ligands can be used to determine their influence on the overall allosteric equilibrium.

The coupling between the spin state and the allosteric equilibrium has been questioned (Philo and Dreyer, 1985); their magnetic susceptibility measurements indicated a small change in the iron spin addition of IHP, which induces a shift towards the *T*-state. It was previously thought that IHP induces a nearly complete transition to the *T*-state for most high spin or mixed spin ligands. This appears to be the case for some root effect hemoglobins, each as carp Hb (Messana et al., 1978); however, the changes in both the allosteric and spin equilibria appear to be smaller in human Hb. Thus, the coupling may still be operational, although the IHP induced changes are reduced.

In this study we consider another method of investigating the contribution of the individual heme groups towards the overall allosteric equilibrium. We consider different levels of oxidation to study the influence of 1, 2, or 3 met hemes, and can then vary the ferric ligand to see whether the local change induces a shift in the overall allosteric equilibrium. Rather than using the absorption changes (upon addition of effector) for the completely met form, which cannot be calibrated, we use the oxygen and CO binding properties to determine the allosteric equilibrium. The *R* to *T* equilibrium for the completely oxidized form can then be obtained by extrapolation.

The present study indicates that the *R* to *T* transition induced by IHP has been overestimated. Whereas Hb molecules within a single crystal are probably all constrained to take on the *R* or *T* conformation, solution studies might show an equilibrium between the two forms.

The present results also provide information on the *R* to *T* equilibrium at the triply liganded level. In analogy to the triply liganded species, we produced triply met tetramers. This allows observation of ligand binding properties to the single ferrous heme (Marden et al., 1991) and a study of the dependence of the allosteric equilibrium on the spin state of the other three subunits.

MATERIAL AND METHODS

Purified stripped human Hb *A* was prepared as previously described (Kister et al., 1987) and stored in the oxy form in liquid nitrogen. Two

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methods were used to oxidize the samples. The first was by addition of ferricyanide, followed by removal of excess ions on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. The second method was through autoxidation at a reduced oxygen partial pressure at 37°C for 48 h at pH 6.5, 50 mM Bis-Tris buffer, 100 mM NaCl, and 20 μg/ml chloramphenicol (to prevent bacterial growth). Ferric ligands were fluoride (20 mM KF), cyanide (2 mM KCN), nitrite (2 mM $NaNO_2$), azide (2 mM NaN_3) and 2 mM Imidazole (Im).

The percent oxidation was calculated using the known absorption spectra at a given pH for the oxy, deoxy, and met forms (Benesch et al., 1973; Van Assendelft and Ziljstra, 1975). At high levels of oxidation, a second measure of the percentage ferrous is the change in absorption upon oxygen or CO binding.

Oxygen equilibrium curves (OEC) were measured with a Hemox analyser (TCS Medical Products Co., Huntington Valley, PA) as previously described (Kister et al., 1987). Typical samples were 60 μM (total heme) for ferrous Hb samples, but 300 μM at 90% oxidation to maintain a sufficiently large oxygen binding signal.

Flash photolysis measurements were made with 95% met-Hb samples (200 μM total heme), equilibrated with 0.1 atm CO, 50 mM phosphate buffer at pH 6.5. MetHb shows a slow reduction by CO (Bickar et al., 1984; Young and Caughey, 1987); ~1 h is required for a change from 98% met to 90% met. A typical series of measurements consisted of monitoring the photolysis signal amplitude until the desired percent met was obtained. The kinetics were then recorded for the aquo-met sample, first without and then with effector (IHP); finally the alternate ferric ligand was added and the kinetics were again measured. Because all three measurements could be made within a few minutes, the percent met was little changed. In another series, the second ferric ligand was added before the effector.

Effector concentrations were 1 mM for IHP (Sigma Chemical Co., St. Louis, MO) and 0.2 mM for L345, 2-[4-(3,4,5-trichlorophenylureido)phenoxy]-2-methylpropionic acid, a more potent derivative of bezafibrate (Lalezari et al., 1990).

Simulations

In the original two-state model (Monod et al., 1965), the allosteric equilibrium is:

$$T_i/R_i = Lc^i, \tag{1}$$

where L is the allosteric equilibrium coefficient for the deoxy form and i is the number of ligands bound. Each ferrous ligand shifts the equilibrium by a factor $c = K_R/K_T$; we use an analogous parameter m for the met hemes. For example, tetramers with two ferric hemes will have equilibria Lm^2, Lm^2c, and Lm^2c^2 for the forms with 0, 1, and 2 ferrous ligands (oxygen or CO) bound.

The first step is to construct the distribution of tetramers having zero to four ferric hemes. For identical subunits, the binomial distribution gives the fraction (F) of each species:

$$F_j = 4!(1-f)^{(4-j)} * f^j/[j! * (4-j)!], \tag{2}$$

with f the total fraction of oxidized hemes and j the number of ferric hemes per tetramer.

The second step is to calculate the ligand saturation for each partially met species. The two-state formalism is used, with α = [ligand]/K_R, where K is the equilbrium dissociation coefficient. For tetramers with j ferric hemes, the fraction ligand saturation of the ferrous hemes is:

$$Y_j = \frac{\alpha * (1+\alpha)^{(3-j)} + L(m)^j(\alpha c)(1+\alpha c)^{(3-j)}}{(1+\alpha)^{(4-j)} + L(m)^j * (1+\alpha c)^{(4-j)}}. \tag{3}$$

For the special case when $m = c$, the formula with two ferric hemes is the same as that for symmetrical Hb valency hybrids (Szabo and Karplus, 1975). The OEC for each species is then weighted by the fraction of each species and by the number of ferrous hemes per tetramer to obtain the overall fraction ligand saturation:

$$Y = \sum_{j=0}^{4} (4-j) * F_j * Y_j. \tag{4}$$

Simulations for a series of curves at different oxidation levels were made by varying only the allosteric equilibrium, using a nonlinear least-squares fitting (Kister et al., 1987). The standard error per point was <0.01 for ferrous Hb and tended to increase with the percentage met, in part because the signal to noise is lower, to ~0.02 at 90% metHb.

For the kinetic simulations, the R, T species reequilibrate after photodissociation; however, the dimer-tetramer reactions are slow compared to the CO rebinding time scale. A large contribution (10%) of nonallosteric forms such as dimers was needed to fit the kinetic results. Use of effectors decreased this static nonallosteric contribution.

At high percentages of oxidation, the ferrous ligand binding signal is predominantly due to tetramers with three ferric hemes; for example, at 95% met, the relative populations are 0, 0.05, 1.4, 17.1, and 81.5% for the forms with 0, 1, 2, 3, and 4 met hemes; this gives percentage ferrous ligand binding signals of 0, 1, 13, 86, and 0%, respectively.

RESULTS

Equilibrium measurements

Oxygen equilibrium curves at various levels of oxidation, with CN bound to the ferric hemes, are shown in Fig. 1. The fraction saturation Y refers only to the ferrous hemes: for example, at 80% overall oxidation, $Y = 0.5$ represents 10% oxygen bound and 10% deoxy hemes (and 80% CN bound). Simulations were made with the modified two-state model (*solid lines* in Fig. 1), with $c = m$, meaning that each CN ion makes the same contribution as an oxygen for calculating the allosteric equilibrium. For example, tetramers with one oxygen bound and three deoxy hemes have the same probability of being in the R or T state as a tetramer with one CN bound and three deoxy hemes; tetramers with three CN ligands will show the same oxygen affinity (R-state) as for the fourth ligand in a ferrous tetramer. This leads only to a left shift in the OEC relative to ferrous Hb, because with CN as ligand, there is always a larger fraction R-state for the partially oxidized tetramers.

OEC with fluoride as the ferric ligand are shown in Fig. 2. The left shift at low levels of oxygenation is smaller than for the case with CN as ferric ligand, and the OEC at different levels of oxidation intersect. Simulations required $m/c = 3.5$, indicating that triply fluoro-met tetramers have an allosteric equilibrium shifted (by a factor of 43) towards the T-state relative to triply oxygenated Hb; this leads to the crossover of the

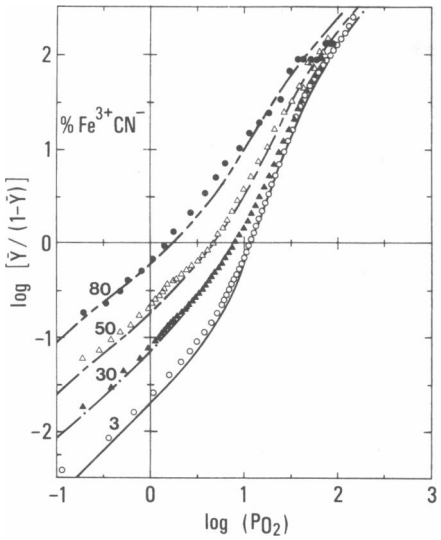

FIGURE 1 Oxygen equilibrium curves (OEC) for the percent cyanometHb shown, 50 mM Bis Tris buffer at pH 6.5, 0.1 M NaCl, 2 mM NaCN, 25°C. The symbols represent one of six measured values; the solid lines are simulations using a modified two-state model where ferrous ligands and ferric-CN hemes have an equal effect on the allosteric equilibrium ($L = 1.3 \times 10^6$, $m = c = 0.0066$, $K_R = 0.33$ mm Hg); the fitting was constrained to a single value of the allosteric parameters for the family of curves at different oxidation levels.

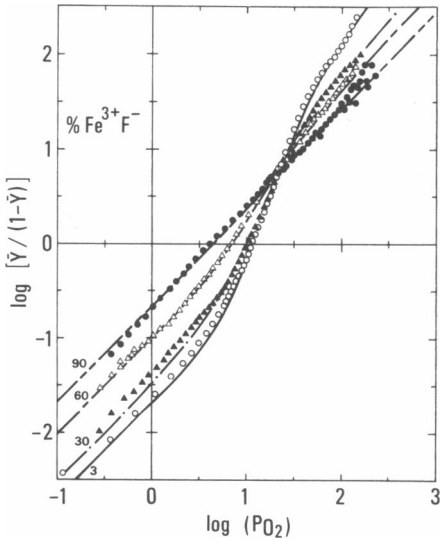

FIGURE 2 OEC for different percentages of fluorometHb at pH 6.5, 20 mM KF, 25°C. Each high-spin ferric ligand causes a weaker shift (factor of $1/m$) towards the R-state, as compared to oxygen ($1/c$). Solid lines are simulations of the generalized two-state model, with c = 0.0066, m/c = 3.5, $L = 1.3 \times 10^6$, $K_R = 0.33$ mm Hg.

OEC near 75% oxygenation due to the increased T-state contribution in the region of the upper asymptote.

OEC for 90% metHb with CN as the ferric ligand are shown in Fig. 3. At this high level of oxidation the signal is mainly for tetramers with a single ferrous heme; the curves therefore show little cooperativity. For all metHb derivatives, there is a shift towards lower oxygen affinity upon addition of effectors. The combination of IHP and L345 induce additive shifts in the oxygen affinity. For triply cyano-met samples, use of both effectors shifts the oxygen affinity to nearly normal (ferrous) T-state values (Fig. 3).

Kinetic studies

Kinetics for the recombination of CO to 95% met Hb are shown in Fig. 4. The biphasic kinetics are characteristic of the Hb tetramer, with the slow phase corresponding to recombination to T-state Hb. For samples equilibrated with 0.1 atm CO, the R-T transition is rapid compared to ligand rebinding; the allosteric equilibrium is thus established after the photodissociation and the fraction slow is a measure of the amount of T-state. Tetramers with the high-spin ligands fluoride or water bound to the ferric subunits showed the most T-state behavior.

CO recombination kinetics to 95% metHb with IHP are shown in Fig. 5. IHP induces a decrease in the recombination rates and an increase in the T-state behavior (Gray and Gibson, 1971). As for the equilibrium and kinetic results without IHP, the high-spin

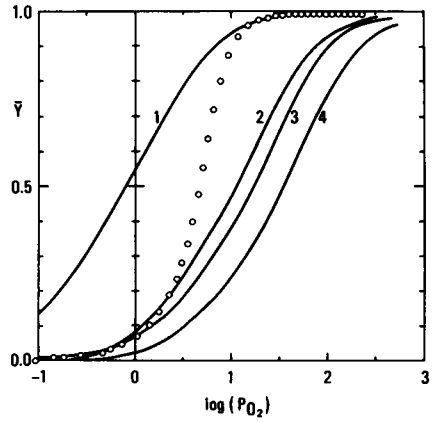

FIGURE 3 OEC for 90% cyanometHb: 2 mM NaCN, pH 6.5, 300 μM total heme, 25°C (curve 1), +0.25 mM L345 (2), +0.3 mM IHP (3), and with both IHP and L345 (4). The OEC for ferrous Hb (o o) in the same solvent conditions is shown for comparison. The effectors IHP and L345 induce an additive shift towards the T-state.

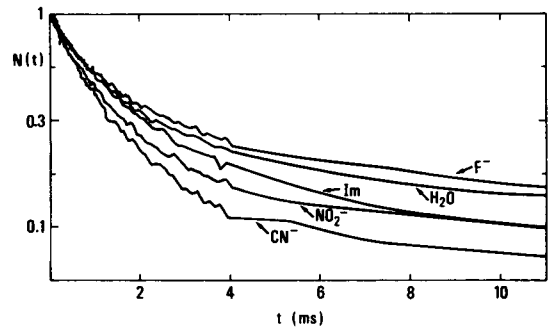

FIGURE 4 Recombination kinetics of CO to 95% met Hb, with various ferric ligands (Im = Imidazole), pH 6.5, 25°C, 200 μM total heme, 0.1 atm CO. The high-spin ferric ligands (F and water) show the most slow phase, characteristic of rebinding to *T*-state Hb. Ferric ligand concentrations were 2 mM, except for F (20 mM).

ligands (water and fluoride) show the most *T*-state behavior. For aquometHb, there is a sharp drop in the fraction *T*-state kinetics above pH 7.2 (data not shown); this results from a combination of changes: (*a*) OH is a low-spin ligand, (*b*) the IHP affinity decreases at higher pH values, and (*c*) the allosteric equilibrium is also pH dependent, with less *T*-state at high pH.

As observed with the OEC, addition of both IHP and L345 produced the maximum amount of *T*-state kinetics. With both effectors present, the CO recombination kinetics of aquo- and fluorometHb samples showed over 95% slow phase; cyano-metHb samples showed 55% slow phase (data not shown).

Analysis

Parameters for simulations with the generalized two-state model are given in Table I. Note that the fraction

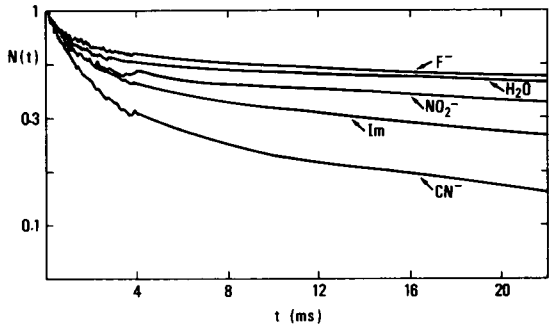

FIGURE 5 CO rebinding to 95% metHb with 1 mM IHP, pH 6.5, 25°C, 200 μM total heme, 0.1 atm CO. As for Hb without IHP (Fig. 4), the high-spin ligands show the most T-state behavior.

TABLE I **Allosteric parameters for partially metHb**

Ligand	IHP	%HS	%*T*		*m*/*c*	%slow	P_{50}	n_{50}
	mM		*j* = 3	*j* = 4			mm Hg	
F	0	95	94	29	3.5	25	6.2	1.1
F	1	96	99	72	2.5	90	98.	1.1
H_2O	0	88	91	18	3.0	22	4.6	1.1
H_2O	1	92	95	52	2.0	86	72.	1.1
N_3	0	12	56	1	1.5	12	1.3	0.9
N_3	1	14	92	8	1.2	55	20.	1.0
CN	0	0	27	0	1.0	9	0.8	0.9
CN	1	0	90	6	1.0	32	20.	1.0

Two-state parameters for ferrous Hb were $L = 1.3 \times 10^6$, $c = 0.0066$, $K_R = 0.33$ mm Hg (without IHP) and $L = 2.5 \times 10^7$, $c = 0.0072$, $K_R = 1.34$ mm Hg (with IHP), at pH 6.5, 0.1 M NaCl, 25°C. Kinetic and equilibrium data were then fit for the partially met Hb samples changing only the allosteric parameter *m* for the met hemes. The overall allosteric equilibrium for a tetramer with *j* ferric hemes and *i* ferrous ligands is Lm^ic^i. %HS is the percentage high spin for metHb, taken from Philo and Dreyer (1985). The %*T* is the calculated equilibrium percentage of *T*-state: fraction $T = Lm^j/(1 + Lm^j)$. The %slow is the percentage of the CO recombination kinetics to 95% metHb occurring with the slow (*T*-state) rate.

high spin and the fraction *T*-state are not necessarily the same, but rather there are four possible states (*R* and *T* conformations, each which may be high or low spin). The overall correlation shows that the spin state is an important parameter in determining the overall R to T equilibrium. The allosteric equilibrium for the triply oxidized species ($j = 3$ in Table I) was calculated as Lm^3, when the fourth (ferrous) subunit is deoxy. We also calculated the percentage *T*-state for fully metHb ($j = 4$), assuming that the fourth met ligand produces the same shift in the allosteric equilibrium as observed for the first three ($T_4/R_4 = Lm^4$). The extrapolated values are consistent with aquometHb being mainly *R*-state (82%) and fluorometHb + IHP as *T*-state (72%), but show that the *R* to *T* transition (±IHP) is not complete.

The parameter "*m*/*c*" is reported in Table I. Relative to the (ferrous) unliganded form, all ligands shift the allosteric equilibrium towards the *R*-state. A value of $m/c = 3$, for example, indicates that the shift towards the *R*-state is three times less than that for the binding of an oxygen. Thus, values of $m/c > 1$ indicate a shift towards the *T*-state relative to the oxygenated form.

We obtained a value of $m/c = 3.5$ for fluorometHb relative to cyanometHb or HbO_2, which should represent the total difference between the high spin and low spin forms. A factor of 4.8 was reported for d/c_0 (Cordone et al., 1990), using a model with a ligation dependent value of *c*; note that we use *m* in place of their parameter *d*.

A least squares error of 0.02 for the fitting of a family

of curves at different oxidation levels, using four parameters, indicates a slight misfit of the data. However, this error is less important than the compensation between the parameters K_R and L. This compensation is especially evident for oxygenation curves which do not make a full allosteric transition, such as for Hb in the presence of strong effectors (Marden et al., 1990) or the present data at high oxidation levels. For example, a sample of predominantly triply met tetramers will necessarily show a noncooperative oxygenation curve; one cannot determine from such an OEC alone both the allosteric equilibrium and the *R*-state affinity. Note that the values reported here are the result of fitting a family of curves at different oxidation levels.

Distribution of states

Identical subunits were assumed for the calculation of the distribution of partially met species. It has been reported that within Hb tetramers the alpha chains oxidized more rapidly than the beta chains (Mansouri and Winterhalter, 1973); however, more recent studies showed little difference in the oxidation rates (Tomoda et al., 1981; Cordone et al., 1990).

A large deviation from the supposed random distribution, such as a mixture of completely ferrous or ferric tetramers, can be tested. At sufficiently high oxidation levels, the triply met form dominates the contribution to the oxygen (or CO) binding signal. The fraction of slow ligand recombination of completely ferrous tetramers shows a dependence on the number of ligands photodissociated. Variation of the laser energy showed little change in the kinetics for 95% oxidized samples, as expected for tetramers with a single ferrous subunit.

A larger error in the distribution of partially met species might be expected near 50% oxidation, if one type of chain was preferentially oxidized. The two methods of oxidation, by ferricyanide or by autoxidation, showed similar results in the oxygen equilibrium curves. Either the two methods yield similar distributions or the curves are insensitive to the differences. Simulations indicate that a chain inequivalence as large as a factor of two in oxidation rate would not significantly change the distribution and therefore the values reported for the allosteric equilibrium, provided the ferrous ligand binding properties are not different.

DISCUSSION

The fraction of Hb in the *T*-state, as evidenced by the low oxygen affinity or slow CO bimolecular rebinding rate, depends on the nature of the ligands bound. Ferrous ligands such as oxygen or CO, and low-spin ferric ligands such as CN, show a similar contribution towards the allosteric equilibrium. The binding of high-spin ferric ligands also shifts the allosteric equilibrium towards the *R*-state, but by a factor three times less than for the low-spin ligands; ferric ligands involving a mixture of high and low-spin states (azide, nitrite, or imidazole) show intermediate values.

Correlation of the *R* to *T* equilibrium with spin state

One problem with a correlation analysis is the lack of intermediate spin values. Use of the ligand CN provides a low-spin reference, while aquo- and fluoromet Hb are predominantly high-spin. Yet there are few examples of mixed spin derivatives to test for a linear relation. This is further complicated by the fact that each ligand may show unique properties, such as imidazole which may involve additional steric factors. Whereas a strict correlation is difficult to prove, we observed that species with more high-spin showed a larger amount of *T*-state behavior. Also, a larger change (±IHP) in the CO kinetics was observed for the nitrite derivative compared to azide, consistent with the magnetic susceptibility measurements (Philo and Dreyer, 1985; Noble et al., 1989) showing a smaller spin change for azidometHb. A correlation between the spin-state equilibrium with absorption properties has previously been reported (Noble et al., 1989).

Effectors

As previously observed in equilibrium and kinetic studies (Marden et al., 1988, 1990; Lalezari et al., 1990), there is an additive shift towards the *T*-state when using multiple effectors. With the ferric hybrids, we again observed a larger shift when using both IHP and bezafibrate derivatives, than with either effector alone. The combination of IHP and L345 induced over 50% slow (*T*-state) kinetics in triply cyanometHb.

The original experimental evidence for the *R* to *T* transition in met Hb was the change in the absorption spectra, but there was no way of calibrating the fraction *R* and *T*. The crystallographic studies give the misleading impression of an all or nothing transition. The present results indicate that the transition is not complete, with a change from 18 to 52% *T*-state upon addition of IHP to aquometHb.

The overestimate of the *R* to *T* transition due to IHP is mainly responsible for the conclusions that the spin and allosteric equilibria were not coupled: whereas the coupling predicted a free energy change on the spin equilibrium of ~1,200 cal/mol, only 100–300 cal/mol were observed (Philo and Deyer, 1985). But they as-

sumed that IHP causes a full R to T transition. Using their spin data for aquometHb, and the present results for the allosteric equilibrium, we calculated a free energy change of 1,200 cal/mol, as predicted by Perutz et al. (1974*a*). Similarly, their data for azidometHb, that show a small change in spin, are compatible with a coupling mechanism after taking into account the small change (7%) in the allosteric equilibrium.

The difference between human and carp Hbs (Noble et al., 1989) is not that the coupling does not work for human Hb, but simply that the effector induced differences are smaller for human Hb. However, use of more than one effector can increase the differences.

The free energy changes (500–800 cal/mol) due to both IHP and Bezafibrate (Noble et al., 1989) are larger than those of IHP alone (250–500 cal/mol), confirming that the allosteric transition induced by IHP is incomplete.

The fraction T-state for fully metHb given in Table I ($j = 4$) was based on extrapolation, using the same factor m for the fourth met heme as measured for the first three. A value of 52% T-state for aquometHb (with IHP) was calculated, indicating that a much larger change of absorption for the R to T transition is potentially observable. To test this conclusion, the absorption spectra were measured in the presence of both IHP and L345; the changes were nearly twice those for IHP alone (data not shown), again confirming that the R to T transition is not complete with IHP alone. Absorption changes occur for a shift in allosteric equilibrium, in spin equilibrium, and due to effector binding; it is difficult to separate these effects to precisely test the dependence on each parameter (Perutz et al., 1974*b*). Although the spectral change may not represent only the R to T transition, the spin change is small for aquometHb (Table I) and the change in absorption was similar for the two effectors, despite different binding sites.

Triply liganded tetramers

It is difficult to determine whether the allosteric mechanism is operational at each ligation level. The tetramers with 0 or 1 ligand are so predominantly T-state ($T_0/R_0 = L = 10^5$ and $T_1/R_1 = 10^3$) that even a factor of 10 shift towards the R-state would not change the observed affinity for the first ligand. The triply liganded tetramers are also difficult to study. The partially met tetramers allow a comparison of oxygen or CO binding data of the ferrous subunit for different ferric ligands.

For equilbrium oxygen binding to ferrous Hb, there is such a low population of triply liganded tetramers that conclusions concerning the allosteric equilibrium for this species are sensitive to slight differences in the oxygenation curves and normalization of the data (Marden et al., 1989). Flash photolysis methods which can photoproduce mainly the triply liganded form are more sensitive to the allosteric equilibrium of this species. Results with strong effectors show that a transition R_3 to T_3 may occur (Marden et al., 1988). These kinetic results are supported by equilibrium data in the presence of strong effectors (Marden et al., 1990; Gill et al., 1989). A modulation technique (Zhang et al., 1990) also shows a substantial signal for the R to T transition under conditions of weak photodissociation where mainly the triply liganded forms are involved.

The present results with three ferric ligands also show T-state behavior. The results at 95% met Hb show that the allosteric equilibrium can still be modified by the choice of ferric ligand or by use of various effectors; this implies that the allosteric equilibrium is still operational at the triply liganded level.

Conclusions

Many T-states of hemoglobin are now known to exist; the oxygen affinity for these T-state varies by over a factor of 50 with different effectors (Marden et al., 1990). It is then of interest to know whether the T-met state has the same properties as the ferrous T-state. This cannot be determined for completely oxidized Hb, but we have shown that triply met tetramers and ferrous Hb have similar oxygen affinities for the R and T-states.

The observed allosteric equilibrium depends on the ferric ligand and is generally correlated with the ferric spin state. We have simulated the data using the ferrous R and T binding affinities and modified only the allosteric contribution of the ferric hemes. Relative to ferrous deoxy Hb, low-spin ferric ligands such as CN make the same contribution as oxygen, that is a shift of a factor of 150 towards the R-state (at pH 6.5, 25°C) for each ligand, whereas a high-spin ferric ligand shifts the allosteric equilibrium by a factor of 40. This results in an observable increase in T-state behavior if low-spin ferric ligands are replaced with high-spin ligands.

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